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Cell Envelope Associations of *Aquaspirillum serpens* Flagella

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Specific regions of the cell envelope associated with the flagellar basal complex of the gram-negative bacterium *Aquaspirillum* (*Spirillum*) *serpens* were identified by studying each of the envelope layers: outer membrane, mucopeptide, and plasma membrane. The outer membrane around the flagella insertion site was differentiated by concentric membrane rings and central perforations surrounded by a closely set collar. The perforations in both the outer membrane and the isolated mucopeptide layer were of a size accommodating the central rod of the basal complex but smaller than either the L or the P disks. The P disk of the complex may lie between the mucopeptide and the outer membrane. Electron microscopy of intact, spheroplasted, or autolyzed preparations did not adequately resolve the location of the inner pair of disks of the basal complex. Freeze-etching, however, revealed differentiation within the plasma membrane that appeared to be related to the basal complex. The convex fracture face showed depressions which are interpreted as impressions of a disk surrounded by a set of evenly spaced macromolecular studs and containing a central "plug" interpreted as the central rod. In thin sections, blebs, which appear to be associated with the flagellar apparatus, were seen on the cytoplasmic side of the plasma membrane. Superimposing the dimensions of the flagellar basal complex and the spacings of the cell envelope layers and using the position of the L disk within the outer membrane for reference, showed that the S disk might be within and the M disk beneath the plasma membrane. A tentative model was developed for comparison with that based on the structure of the *Escherichia coli* basal complex.

It is now well understood that bacterial flagella are comprised of three morphologically distinct regions: the helical filament consisting of closely packed flagellin subunits; the serologically and structurally distinct hook, which is usually set off at an angle; and the basal complex, which is inserted into the envelope layers.

High-resolution electron microscopy of the basal complex after dissolution of the envelope and of partially damaged structures led to a model of the flagellar insertion (7, 8). Four disks were clearly identified, arranged as two pairs on a central rod: an outer pair (L and P) appeared connected near their periphery because of lack of penetration of negative stain; the inner pair (S and M) were closer together than the outer pair. The disks were designated according to their presumed attachments; L to the lipopolysaccharide-containing membrane; P to the peptidoglycan layer; S for "supramembrane"; and M to the cytoplasmic membrane. Studies in many laboratories show that these features of the basal complex are common to most flagellated gram-negative bacteria.

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The specific attachments of each of the disks to the cell envelope were determined by DePamphilis and Adler (9). They purified the outer membrane of *Escherichia coli* and observed that the disk closest to the hook region was attached to this membrane; this was also observed when spheroplasts were osmotically shocked. The inner M disk, on the other hand, seemed to remain embedded in the plasma membrane with the S disk just outside it and unattached to any structure. Thus the evidence of the attachment of the two disks was apparently direct. Futhermore, the P disk was considered by DePamphilis and Adler to be in register with the peptidoglycan layer because the dimensions assigned to the appropriate layers of the cell envelope (10, 11) matched those of the basal complex.

A critical question as to whether or not an essential component of the apparatus was lost during preparation cannot be satisfactorily answered. The possibility still exists that a fragile component at the flagellar base is destroyed or modified in the isolation procedure.

This paper provides additional direct evidence for structural associations of membrane and wall

with the elements of the basal organelle of *Aquaspirillum* (*Spirillum*) *serpens*. This organism has the advantage of demonstrable structures in the outer membrane associated with the insertion site of the basal complex (concentric membrane rings [CMRs]; 6) and a restricted area of insertion for the bundle of flagella at each pole (16, 20). Isolation of the outer membrane, the mucopeptide layer, and the cytoplasmic membrane and identification of the specific regions of each penetrated by the basal complex have allowed modification of the currently accepted model for flagellar insertions. These modifications take into account the respective dimensions of the basal complex, the disks of the basal complex, the depressions and perforations of the envelope layers at the flagellar insertion site, and the section profiles. Although uncertainties remain, an anatomical model of the basal complex and its associations with the cell envelope is presented.

MATERIALS AND METHODS

Bacteria, media, and growth conditions. *A. serpens* VHL (from the Culture Collection of the University of Western Ontario, U.W.O. 368) was chosen because it lacks the superficial protein arrays or RS layer (3, 4) which would have been a nuisance in the isolation and characterization of the outer membrane. In some experiments involving embedding of spheroplasts for electron microscopy, the wild-type strain VHA was used because the profile of the outer membrane could be identified by the RS layer (3, 4). Both strains display the structural profile of a gram-negative cell envelope (1, 10, 11, 17). Cells were grown in liquid medium consisting of, per liter, 1.0 g of yeast extract (Difco), 1.0 g of tryptone (Difco), 0.5 g of sodium acetate anhydrous, and 0.25 g of magnesium sulfate (pH adjusted to 7.6). Cultures were incubated at 30°C with shaking, giving a 60-min generation time. *Spirillum volutans* Wells (kindly supplied by N. R. Kreig, Virginia Polytechnic Institute, Blacksburg) was grown as suggested by Wells and Kreig (22) in the same medium as *A. serpens* but in an atmosphere of 6% oxygen–94% nitrogen at 30°C.

Preparation of membranes. The conditions for spheroplasting and for the separation and identification of inner and outer membranes have been described previously (6).

Preparation of murein sacculi. Murein sacculi were isolated from *A. serpens* and from *S. volutans* by modifying the procedure of Weidel et al. (21). Stationary-phase cells were harvested by centrifugation (10 min at 1,085 × *g*, room temperature) and twice washed in distilled water. The pellet was then suspended in a solution of 4% sodium dodecyl sulfate in distilled water and heated in a boiling water bath for 30 min. The remains of the envelopes were recovered by centrifugation (15 min at 27,000 × *g*, 4°C) and washed six times in distilled water. The washed pellet was then extracted with an aqueous solution of 80% (wt/wt) phenol for 2 h at room temperature (22 to 24°C) and again washed as described above. Finally, the suspensions were incubated in 0.01 tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.6) containing 250 μg of trypsin per ml for 12 h at 37°C. After three washes in distilled water, the murein sacculi were negatively stained and examined by electron microscopy.

Isolation of intact flagella. Intact flagella from *A. serpens* were isolated by the procedure of DePamphilis and Adler (7) in which spheroplasting preceded lysis by a nonionic detergent (2% Triton X-100); differential ammonium sulfate precipitation and dialysis against tris(hydroxymethyl)aminomethane-Mg^{2+} were sufficiently gentle to obtain intact flagella. Despite faithful reproduction of this procedure, the yield of intact flagella with attached hook and basal complex was very low, and *A. serpens* spheroplast membranes were less sensitive than those of *E. coli* to disruption by Triton X-100 and ethylenediaminetetraacetic acid. For this reason, membrane vesicles contaminated the flagellar preparations. However, intact flagella were present and served for accurate measurements of the fine structure of the basal complex in negatively stained preparations.

Electron microscopy. (i) Negatively stained preparations. Negatively stained preparations were made by allowing a 5-μl sample to partially dry on the surface of a Formvar-coated, carbon-stabilized, 200-mesh copper grid, then removing excess liquid with bibulous paper, breaking a film of the stain in a 3.5-mm loop over the surface of the grid, and allowing it to dry. Solutions of 1% ammonium molybdate in 0.05 M ammonium acetate (pH 7.5), 1% potassium phosphotungstate (pH 7.5), or 1% uranyl magnesium acetate were used for staining.

(ii) Fixation and embedding. Samples were fixed by suspension in 2% glutaraldehyde buffered with 0.10 M sodium cacodylate (pH 7.5) for 1 h at room temperature. After washing in buffer, the material was enrobed in agar, cut into small blocks, and postfixed with 1% osmium tetroxide for 5 h and then with 1% uranyl acetate for 1 h at room temperature. Embedding was in Epon 812. Sections were picked up on 200-mesh copper grids covered with Formvar/carbon and were stained with 1% uranyl acetate and with lead citrate.

(iii) Freeze-etching. Vesicle preparations or whole-cell preparations, suspended in 20% glycerol as cryoprotectant, were concentrated by centrifugation (27,000 × *g* for 20 min, room temperature). A portion of the pellet was deposited on copper disks, frozen immediately in Freon 22, and stored in liquid nitrogen. Freeze-etching was carried out according to the method of Moore (15) by using a Balzers apparatus (model BA510 M, Balzers AG, Liechtenstein). The fractured samples were etched for 1 min and shadowed with platinum and carbon. The resultant replicas were washed with distilled water, concentrated H_2SO_4, and acid hypochlorite solution, then transferred to 200-mesh copper grids.

(iv) Microscopy. A Philips EM-300 electron microscope operating at 60 kV, with an objective aperture of 50 μm, was used. When the Philips goniometer stage was used, the sections were tilted in a plane at right angles to the plane of the plasma membrane and,

where possible, micrographs were taken at 6° intervals of tilt for as much at +36° and −36° from the minimum membrane profile. Micrographs were taken on 35-mm Kodak Fine Grain Positive Film.

RESULTS

Structure of the basal complex. The initial objective was to establish the structure of the basal complex in *A. serpens* and to compare it with the established *E. coli* model (9) by using preparative methods similar to those used in the latter study and minimizing shearing proximal to the hook region. The basal organelle (Fig. 1) consisted of two pairs of disks. The outer pair, L and P, appeared very closely apposed, and were approximately 18 and 21 nm in diameter, respectively. The inner pair (S and M) appeared to retain some adherent membrane, which may have exaggerated their diameters (ca. 28 and 31 nm, respectively). The disks were connected by a rod 10 nm in diameter, which was a value important to assessing the perforations in wall layers. Fibrillar projections were present at the base of the M disk on the isolated complex; these may be relics of the insertion into plasma membrane or part of some additional but poorly preserved structure.

Dimensions of wall perforations. Perforations were observed in the various wall layers; measurements of these were made to allow comparison with the dimensions of the traversing structures of the basal complex.

(i) Outer membrane. Polar envelope fragments (hemispherical ends) showed a set of clearly defined 12-nm-diameter "holes" which filled with negative stain. CMRs, which have been shown to be attached to the innerside of the outer membrane (6), provided an additional component surrounding the insertion of the basal complex. They formed a plate-like array of protein subunits assembled in up to seven rings with a maximum diameter of 90 nm (Fig. 2). The innermost circle of the CMRs seemed to be wider and better defined than the outer rings and formed a collar around the perforation in the outer membrane.

(ii) Site and thickness of CMRs. The anatomical site of the CMRs on the inside of the outer membrane was directly demonstrable in sections of lysozyme spheroplasts fixed after being gently lysed by dilution or by very brief (5-s) sonic oscillation. Separation of the envelope layers was attained while the basal complex remained in situ as an "adhesion zone" (2). This allowed visualization (Fig. 3A) of a highly scattering layer of an overall width of 60 to 70 nm, which approximated to but was smaller than the maximum diameter of CMRs on the inside of the outer membrane in negative stains, and about 5 to 7 nm in thickness. The latter figure

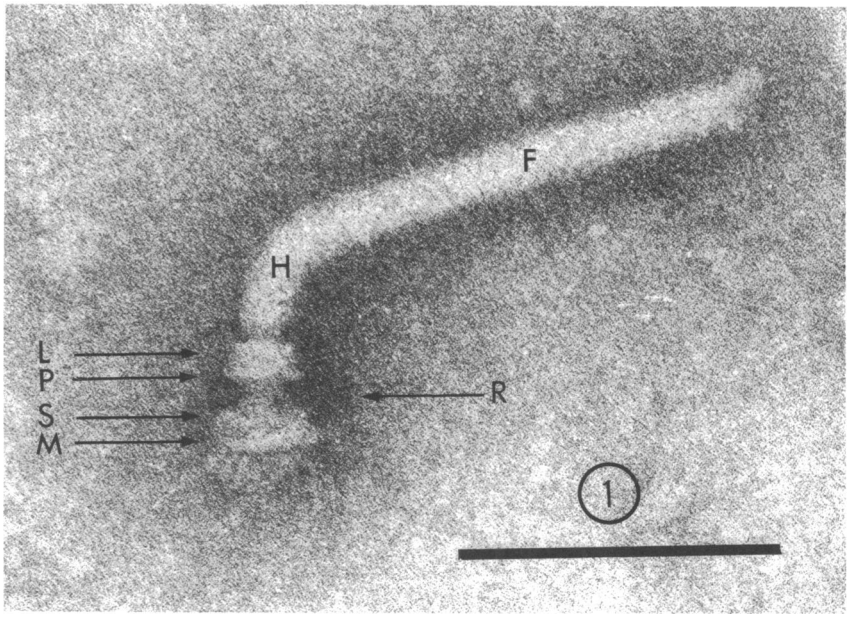


FIG. 1. *Intact organelle: hook (H), filament (F), and basal complex from A. serpens. The basal complex is composed of four disks (L, P, S, and M) set on a central rod (R) connecting the outer pair to the inner pair of disks. The rod emerges from the basal complex to join the hook. Negatively stained with 1% phosphotungstic acid. Bar, 100 nm.*

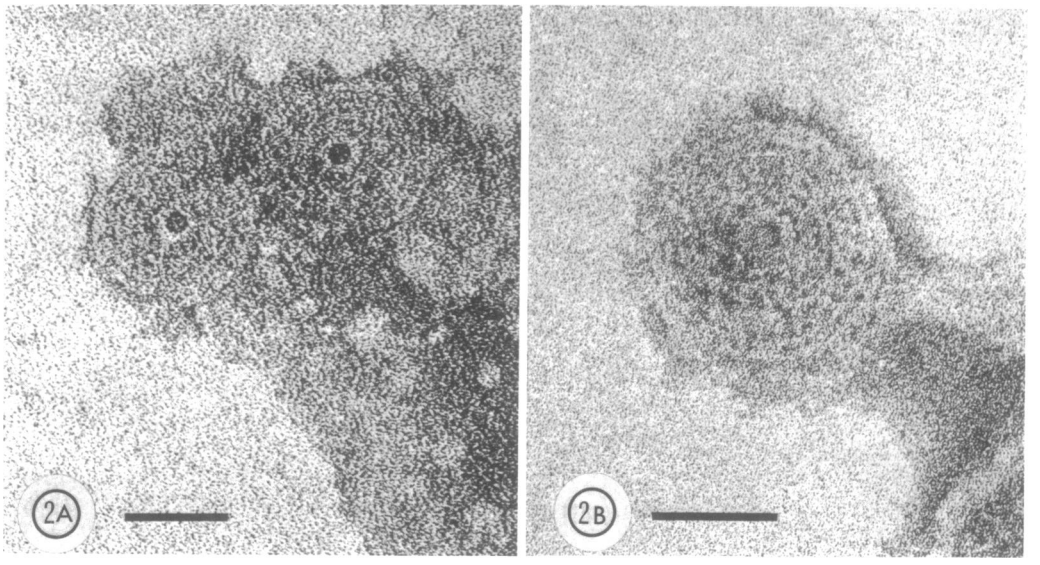


FIG. 2. *Examples of CMRs from preparations of outer membrane. There is uniformity of structure, and each ring of the array is evenly spaced. The 12-nm-diameter central hole into which stain collects is surrounded by a collar and, in the center, the hollow tube form of the central rod is seen (A). Negatively stained with 1% phosphotungstic acid (A) or 1% ammonium molybdate (B). Bar, 50 nm.*

might be exaggerated due to contraction of the CMR plate in fixed preparations. It was not embedded in the membrane, confirming the previous impression that the attachment was superficial (6). Tilting of the specimen in a goniometer stage substantiated the concept that this restricted layer represented the profile of the CMRs (Fig. 3B), because the profile of the highly scattering layer became ovoid at ca. 24° tilt. Because the section, in all probability, exceeded 60 nm in thickness, it is likely to have contained all or most of the CMR, which would present its broader aspect on tilting. The micrographs both represent only slight tilting with respect to the membrane.

(iii) Murein sacculus. Perforations were also observed only at the poles of negatively stained murein sacculi. They were 12 to 20 nm in diameter, slightly larger (15 nm average) than those in the outer membrane but much smaller than any of the disk diameters. Because of the small number (<15) of flagella at each pole of *A. serpens* VHL, the more richly flagellated (50 to 100) *S. volutans* (Wells strain) was used to prepare murein sacculi; it provided the same result (15 ± 5 nm, range 10 to 20 nm). This organism also possessed CMRs on the inner face of the outer membrane and a basal complex of structure and proportions similar to those of *A. serpens*. Shadowed replicas of the poles of sacculi as well as ammonium molybdate and uranyl magnesium acetate used as negative stains were not effective in demonstrating the polar perforations. However, 4% phosphotungstate allowed a clear demonstration and furthermore showed an electron-transparent "halo" surrounding each perforation (Fig. 4). In both organisms, the number of polar perforations was roughly equivalent to the number of flagella, and no perforations were observed along the lateral walls. The variability in diameter may be the expression of some elasticity of the sacculus. The diameter of the halo was determined with less precision as 35 nm, because of indistinct margins; this may be interpreted as the impression of the P disk compressing a portion of structure making it less penetrable to stain, a thickening of the layer, or a combination of these effects.

Insertion of the basal organelle into cytoplasmic membrane. A variety of preparations proved valuable in assessing the insertion of the basal organelle into the cytoplasmic membrane.

(i) Autolyzed preparations. Autolyzed preparations of *A. serpens* were the most useful for electron microscopic examination when the cells were devoid of cytoplasm. A culture was left standing at room temperature for several days and was harvested by centrifugation, twice washed with distilled water, and negatively stained with ammonium molybdate. Some lysed cells retained the flagellar hook and basal complex firmly attached and connecting the outer membrane and plasma membrane of the cell envelope (Fig. 5), despite considerable retraction of the cytoplasmic membrane at regions other

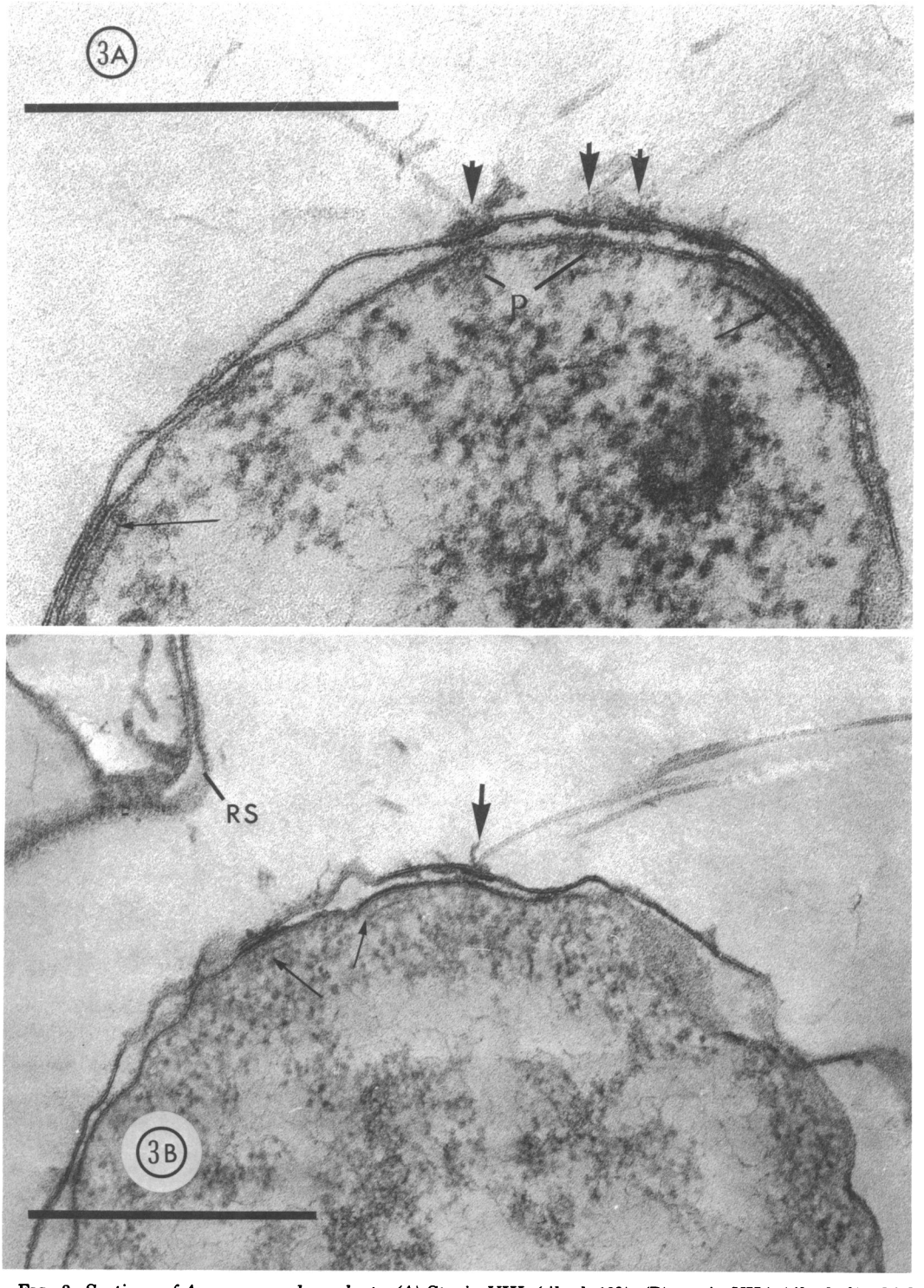
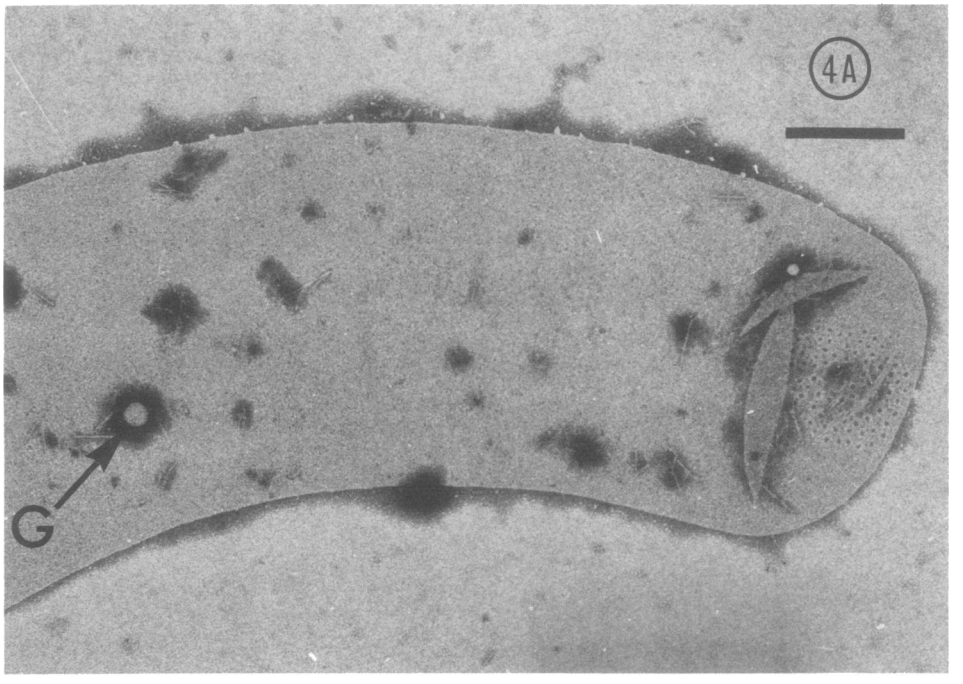
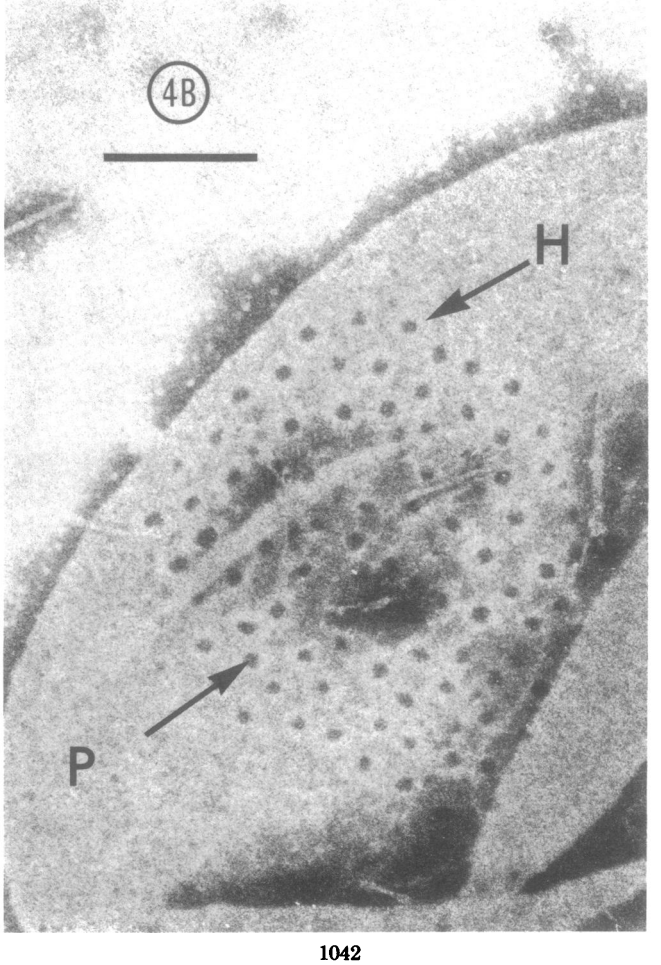


FIG. 3. *Sections of A. serpens spheroplasts. (A) Strain VHL (tilted, 18°); (B) strain VHA (tilted 6°) which has lost the RS layer except on the portion of wall at upper left. The profile of the CMRs shows on the underside of the outer membrane at insertion sites marked by flagella (big arrows). There are collections of staining material on the cytoplasmic side of the sites, which appear to include some ribosomes. The protuberance (P) in (A) seems to separate the flagellar site from the ribosomes and appears to form a small compartment. Note the independence of polar membrane (small arrows). Bar, 0.5 μm.*


4A
G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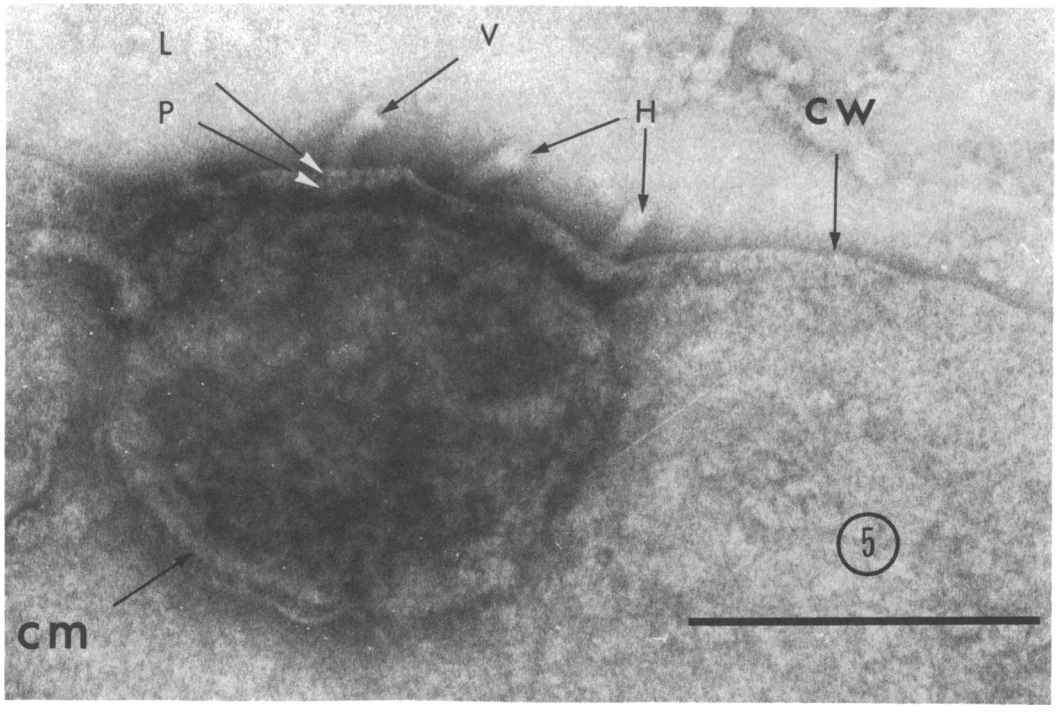

FIG. 5. *Autolyzed A. serpens showing retraction of the cytoplasmic membrane (CM) from the cell wall layers (CW), forming an interior vesicle. Flagellar hooks (H) project from the cell surface, each displaying a V-shaped distal tip (V). The form of the wall suggests a rigid zone, perhaps conferred by CMRs in the wall lateral to the insertion of each hook. The outer pair of disks (L, P) appear to be within the envelope layers, but the placing of the inner pair of disks relative to the cytoplasmic membrane is unclear. Negatively stained with 1% ammonium molybdate. Bar, 200 nm.*

than the polar cap. These preparations demonstrated that: (a) the outer membrane enclosed the L ring; (b) the CMRs were clearly associated with the external membrane and could be discerned in profile by the regular spacing of subunits beside the basal complex; (c) the contour of outer membrane was slightly infolded over the site of the CMRs, which appeared to confer some rigidity to the area at which the flagellum emerged; and (d) the S and M disks appeared to be in contact with the plasma membrane, but their precise attachments were unclear.

Electron microscopic study of these autolyzed preparations yielded information to the observer on the most probable "native" orientation of cell envelope and basal complex; micrographs were difficult to obtain because of the nature of the preparation and stain retention. Actively growing cultures were not amenable to these observations. The need to disrupt the cells by procedures such as sonic oscillation usually sheared off both filament and hook, and often disrupted the basal complex beyond recognition.

(ii) Membrane vesicles. Membrane vesicles, prepared by the isolation of envelope components, were certainly helpful in studies of CMRs in association with outer membrane (6). However, extensive study by negative staining of the plasma membrane vesicles generated in those experiments revealed no structural features that could be related to the basal complex.

(iii) Freeze-etched preparations. Freeze-etched preparations with cleavages through areas of the polar region were rare and hard to find, but when found they often showed a number of structures in the same field (Fig. 6). Cleav-

FIG. 4. *Murein sacculus of S. volutans. (A) The giant bag-shaped macromolecule is devoid of adhering membranes; some poly-β-hydroxybutyrate granules (G) are trapped within. The sacculus has collapsed and shows pleats at the pole. Stain-containing perforations are found only at the pole. Stained with 4% phosphotungstic acid. Bar, 0.5 μm. (B) Higher magnification of the pole of sacculus in (A), displaying perforations (P; diameter, ca. 15 nm) in the murein sacculus through which the central rod passed. A halo (H; diameter, 35 nm) of lower electron-scattering power surrounds each perforation. Variability in diameter may be the expression of some elasticity of the sacculus. Bar, 200 nm.*

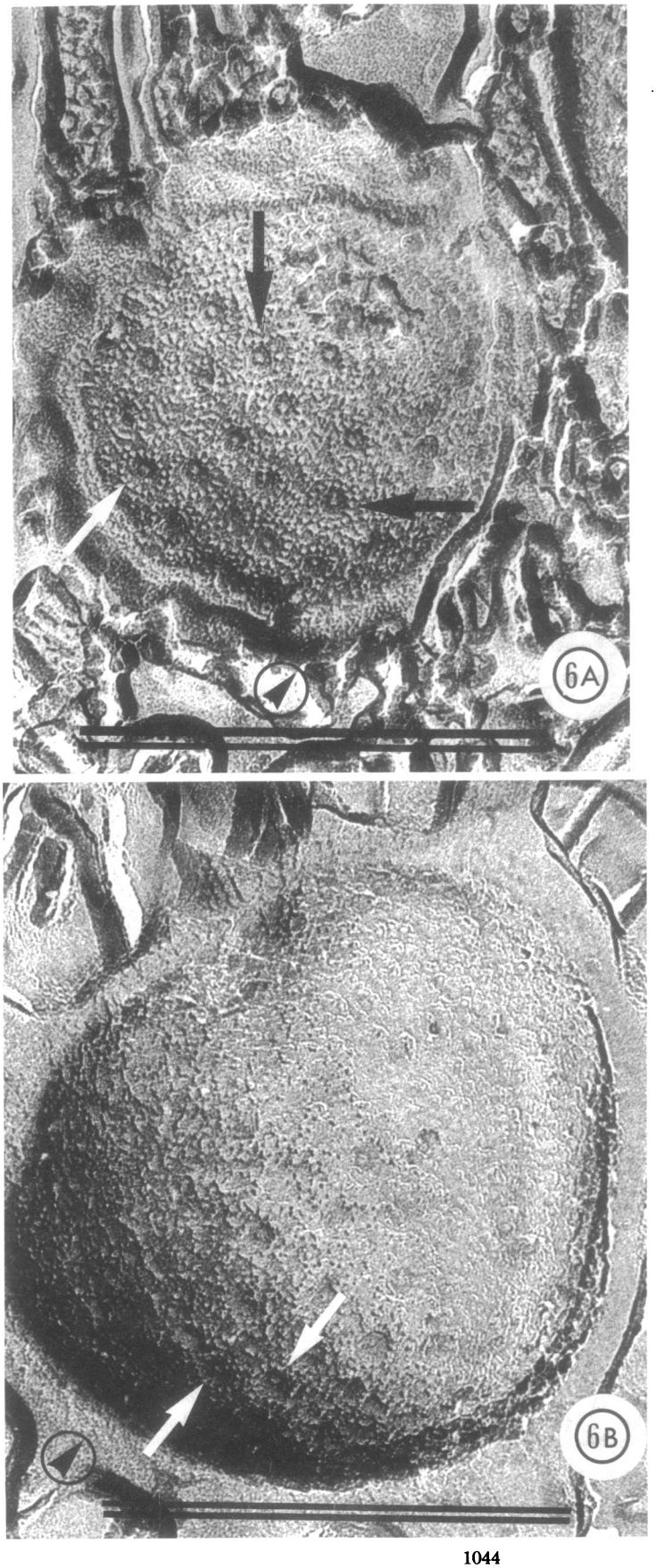

FIG. 6. *Replica of two polar regions of a freeze-etching preparation of A. serpens demonstrating convex cytoplasmic membrane fractures. The circular depressions (diameter, 23 nm) in these cleaved surfaces could accommodate one of the disks of the basal organelle. A central plug (diameter, 10 nm) in each depression correlates with the diameter of the central rod of the basal complex. Many depressions are surrounded by a circlet of small studs (arrows), about 15 in number (range 14 to 16). Circled arrowheads indicate direction of shadowing. Bar, 0.5 μm.*

ages were confined to the plasma membrane (19), and other interfaces could not be identified with certainty except for etching down to the pitted external surface of the outer membrane. The convex fracture face of the polar portion of the plasma membrane showed a set of circular depressions, each with a rounded, raised, central "plug." The periphery of each depression was outlined with a remarkably regular ring of studs at the level of the membrane cleavage (best shown in Fig. 6A). The number of studs is hard to count but seems to be 15 on average and 5 nm in size (notoriously inaccurate on shadowed preparations). We interpret the depressions as disk insertion sites (ca. 23-nm diameter), presuming that the disk was torn out during cleavage. The only remnant of the basal organelle is the central plug (ca. 10-nm diameter), which may be part of the central rod. The concave aspect of the region was not found; we assume that the disk (possibly the S disk) is retained by the outer half on cleavage but is positioned nearly central in the membrane. The circumferential particles seem to be a unique structural feature of the cytoplasmic membrane contributing to the complex.

(iv) Intact cell sections. Sections of fixed and stained intact cells provided another view of the relationship of basal organelle and cell envelope. As with the identification of the holes in the murein sacculus, it was advantageous to use heavily flagellated *S. volutans*. The sections showed (Fig. 7): a tuft of flagella; increased electron density where the flagellum penetrated the cell envelope, due probably to the components of the basal complex, although they could not be identified individually; and an area of electron density beneath the double track of the plasma membrane, manifest as a bulbous component not recognizable by any of the other preparative techniques for electron microscopy. The latter protuberance could not be attributed to deformation of cytoplasmic membrane, which showed regularity beneath those regions from which flagella originated. *A. serpens* also showed, with regularity, a dense but indefinite aggregation of material on the cytoplasmic side of the insertion site (Fig. 3A and B), but a precise definition of structure was not attainable. Each of the micrographs included some portion of the polar membrane, and no continuity or resemblance was observable between this and the internal protuberance. Significant additions to these and similar earlier observations (5, 16) will require more sophisticated techniques. A degree of modification inside the plasma membrane and interpreted as flagella insertion sites was observed in sections of *E. coli* by Bayer (2).

DISCUSSION

Models, especially when associating two complex structures, demand accurate measurements. The first approximation for bacterial flagella was based on the isolated basal complex (7, 8), which permitted accurate measurements of the diameter and thickness of the four disks of the basal organelle. Intercalation of the basal complex into the gram-negative cell envelope was made by superimposing measurements derived from negatively stained preparations onto the thin-section profile from fixed and stained whole cells. Such numerical values are at best an estimate, being subject to some error of measurements; measurements derived from different preparations are not necessarily comparable and are complicated by variations in the composition, environmental pH, and uptake of the stains employed.

The dissolution of the layers of the cell envelope included the possibility, usually overlooked, that some component of the apparatus might have been lost or degraded; the CMRs or a related structure may be such a component. Furthermore, the evidence for the intercalation of the basal complex with each layer of the envelope has thus far been indirect. Those anatomical arrangements put forward in the literature (5, 9, 16, 18) have not specifically considered the complementary regions of wall and membrane which were once sites of flagellar insertions. It is also difficult to imagine a complex motor organelle being entirely contained within a diameter of 25 nm and without recognizable components in the surrounding portion of the envelope. The result of a search for additional components was the description (6) of the CMRs, which have now been observed in a number of *Spirillum* species but not in enteric bacteria or pseudomonads. Moreover, no structural basis for direct association of flagellar bases with the nearby polar membrane has been observed; this membrane was once assigned as a component of the complex (5, 16), and the situation is not yet clarified.

The DePamphilis and Adler model (9) has features that are common to the flagella insertions of many, if not all, gram-negative bacteria, but it is not certain that the locations of all the elements of the basal complex are correct. This study emphasized the difficulty of determining the anatomical associations and especially those of the P, S, and M disks. A major problem is presented by the correlation of spacings determined on unfixed, negatively stained, intact flagella with those obtained from sections of fixed, embedded, and metal-impregnated sections;

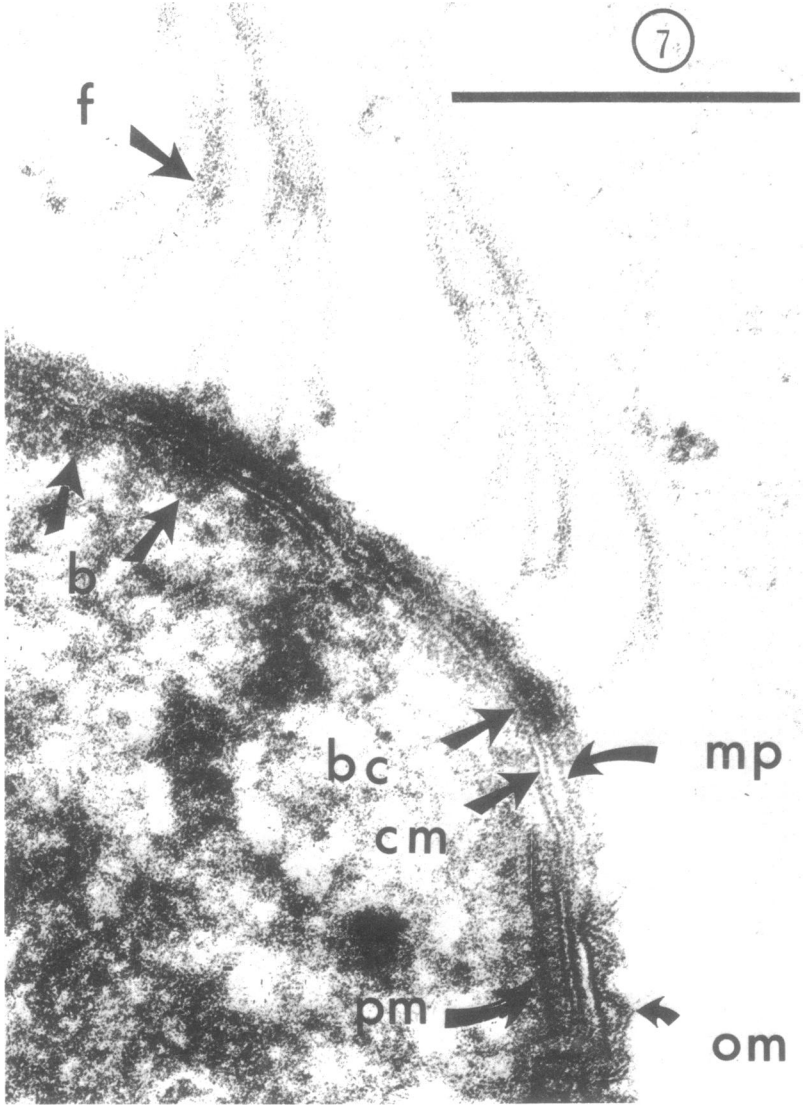

FIG. 7. *Thin section through the polar cap of intact S. volutans. Each flagellum (f) emerges individually through the cell envelope layers: outer membrane (om), mucopeptide (mp), and cytoplasmic membrane (cm). Polar membrane (pm) is also shown. Additional electron density is pronounced in two areas: (i) in the plane of section through the basal complex (bc), so that the disks cannot be resolved; (ii) immediately beneath the cytoplasmic membrane, as a bulbous protuberance (b). The cytoplasm under the flagella insertions had remarkably few ribosomes (see 15). Bar, 0.25 μm.*

both involve uncertainties in the preservation of dimensions. Measurements, treacherous though these may be, indicate that the basal complex requires some 3 to 4 nm more space than the envelope profile would provide without distortion. Therefore, we believe that the details of the model need to be reexamined and the structural parameters of the insertion of this dynamic organelle have to be clarified. As we have found, this is no easy task.

Data obtained from the micrographs of *A. serpens* are displayed in Table 1 together with the parallel information from the studies of *E. coli* (7, 8). The measurements are the best we could make on the material and are not really suitable to statistical treatment, as is also the case for most of the parallel studies available for comparison. The following points have to be considered, despite the speculative aspects of interpretation, in modeling the organelle and representing our interpretations at this stage of our investigation.

TABLE 1. *Dimensions of cell envelope layers and the flagellar basal complex: a comparison*[a]

Dimension	Size (nm) in strain: *E. coli* (DePamphilis and Adler [8, 9])	*A. serpens* (this study)
Diameter of disks (NS)		
L	22.5	18
P		21
S		28
M		31
1 K		22
Diameter of connecting rod (NS)	7	10
Diameter of hole in outer membrane (NS)	ND	12
Diameter (maximum) of CMRs (NS)	NP	90
Diameter of hole in mucopeptide (NS)	ND	15
Diameter of halo in mucopeptide (NS)	ND	35
Diameter of depression on convex cytoplasmic membrane (FE)	ND	23
Diameter of central plug in the depression on convex cytoplasmic membrane (FE)	ND	10
Length of basal complex, "top" to "bottom" (NS)	27	31

Dimension	Size (nm) of strain: *E. coli* (dePetris [10, 11])	*A. serpens* (Murray et al. [17])
Thickness of outer membrane	6.0	7.0–8.5
Thickness of mucopeptide	2.5	4.0–4.5
Thickness of cell wall layers	13.0	13.5–16.5
Thickness of cytoplasmic membrane	6.0	7.5–8.5
Thickness of periplasm (by difference)	4.0	4.0
Thickness of cell envelope (including periplasm)	23.0	25.0–29.0
Thickness of cell envelope		
Inouye (13); t.s.	25.0	
Bayer (1); t.s.	20.0	

[a] NS, Negative staining; FE, freeze-etching; ND, not determined; NP, not present; t.s., thin section. The measurements, like those quoted from others, represent best estimates based on means, but the data in most cases are not suitable for statistical treatment. In some cases, especially membrane and layer thicknesses as well as the length of the basal complex, minimum figures were taken as likely to be closest to accurate.

1. The 12-nm hole in the outer membrane is sufficient to allow for the diameter of the central rod (10 nm); the L disk is accommodated within this membrane. The position of the L disk can be used as a point of reference.

2. The 15-nm perforation in the murein sacculus is a sufficient fit for the central rod, but is clearly too small for the P disk (21 nm) to insert at that level. The measurements suggest that the P disk should be positioned exterior to the mucopeptide layer and may indent it or modify its structure, accounting for the observed halos. A remaining but remote possibility is the identity of the halo with the P disk. However, the basal complex as a whole is soluble in hot sodium dodecyl sulfate, and it seems unlikely that this one disk would survive or would be made of mucopeptide.

3. The depression (23-nm diameter in these shadowed preparations) on the convex fracture face of the cytoplasmic membrane could be the site of either the M or S disk despite the disparities in size. The size of the disks may be exaggerated by adherence of membrane remnants. The cytoplasmic membrane does not show inward displacement at this site in sections (Fig. 3A and B), and superimposition of the measurements (Table 1) suggests that the S disk could coincide with the membrane. The presence of a central plug could be the continuation of the rod inwards to the M disk. Therefore, we are inclined to interpret the depression as the site of the S disk. So far, no negatively stained preparation of spheroplasts has shown a definite supramembrane disk.

4. The 15 circumferential protein studs on the convex cytoplasmic membrane fracture face are the only components of this layer identified thus far that might be functionally associated with the basal complex.

5. Account should be taken of the protuberances that are found on the inside of the cytoplasmic membrane in thin sections; it is suggested that these may form a compartment to include the lowermost M ring.

6. The regular splayed fibrils emanating from the innermost M disk, as seen by negative staining, are of unknown function. They may be membrane associated and be anchorage structures or may be part of the protuberances.

These interpretations are depicted in Fig. 8, which includes the placing of CMR components. We realize the uncertainties of measurement and the hazards of superimposing the electron microscopic data using different preparative techniques and from separate studies. Nevertheless, despite these cautionary notes and the lack of direct evidence in some of the interpretation, we believe it is stimulating to further attempts to resolve the anatomy of this remarkable organelle to attempt a "best fit" of the disks to the

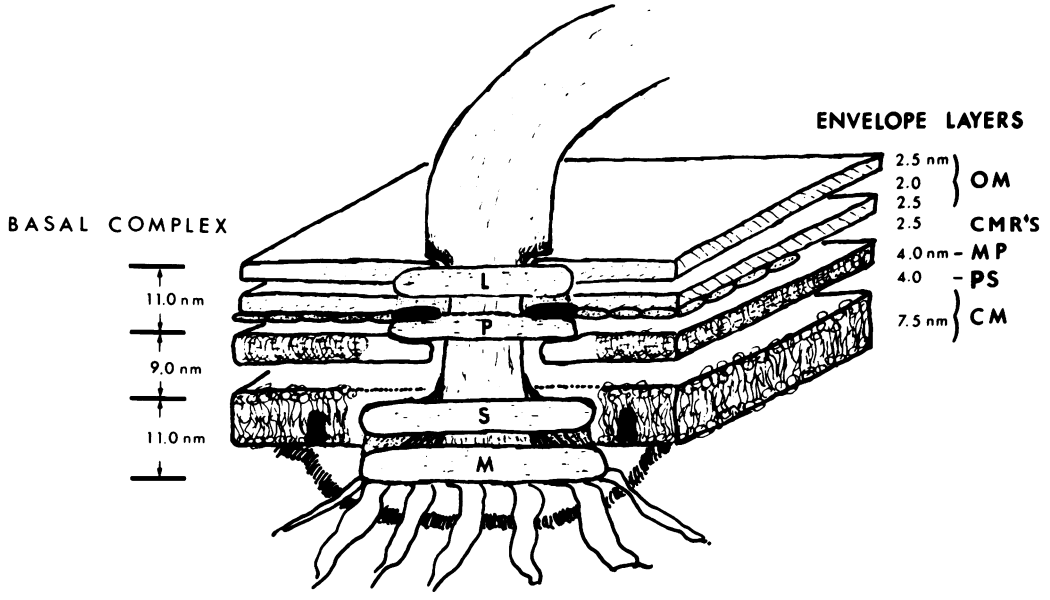

FIG. 8. *A tentative anatomical model to summarize the cell envelope associations of the basal complex derived from this study of A. serpens. The nomenclature for each of the four disks (L, P, S, and M) is that of DePamphilis and Adler (8), and their positions relative to outer membrane (OM), mucopeptide (MP), and cytoplasmic membrane (CM) of the gram-negative cell envelope are displayed. CMRs are oriented on the inside of the outer membrane, and the component macromolecules, unlike the drawing, probably fill the 2.5-nm space assigned to them. The periplasmic space (PS) is shown without macromolecular components. The intra-cytoplasmic membrane studs, the possible sub-cytoplasmic membrane compartment, and the adherent fibrils are included in tentative fashion. The filament, hook, and central rod should be a continuous tube.*

envelope layers and to draw attention to possible disparities in the comparison with data derived from *E. coli*. The model for *A. serpens* should be taken as tentative and speculative until such time as techniques of preparation and measurement allow direct evidence for structural details.

An attractive feature of structural models such as this one is the stimulus to correlate morphogenetic information. There is evidence that monomeric flagellin protein is synthesized on ribosomes (14), and subsequently it must somehow be directed towards the cell envelope for "export." Since flagella are also known to extend in length by addition of subunits at their distal end (12), a mechanism for the transport of flagellin must be invoked. The inner M disk, the fibrils, or another associated structure, situated at the cytoplasmic interface of the membrane, could act as a nucleating center for hook protein and flagellin. The protein could be directed towards this acceptor region of the basal complex and enter the hollow rod and filament on the way to assembly at the tip.

The observations and interpretations presented here constitute a challenge to test currently accepted generalizations on the cell envelope associations of bacterial flagella. It is hoped that the study of a variety of bacteria will continue to expand the understanding of such relationships and eventually lead to a generalized anatomical model.

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LITERATURE CITED

1. **Bayer, M. E.** 1974. Ultrastructure and organization of the bacterial cell envelope. Ann. N.Y. Acad. Sci. **235**:6–28.
2. **Bayer, M. E.** 1975. Role of adhesion zones in bacterial cell-surface function and biogenesis, p. 393–427. *In* A. Tzagoloff (ed.), Membrane biogenesis. Plenum Press, New York.
3. **Buckmire, F. L. A., and R. G. E. Murray.** 1970. Studies on the cell wall of *Spirillum serpens*. I. Isolation and partial purification of the outermost cell wall layer. Can. J. Microbiol. **16**:1011–1022.
4. **Buckmire, F. L. A., and R. G. E. Murray.** 1973. Studies on the cell wall of *Spirillum serpens*. II. Chemical characterization of the outer structured layer. Can. J. Microbiol. **19**:59–66.
5. **Cohen-Bazire, G., and J. London.** 1967. Basal organelles of bacterial flagella. J. Bacteriol. **94**:458–465.
6. **Coulton, J. W., and R. G. E. Murray.** 1977. Membrane-associated components of the bacterial flagellar appa-

ratus. Biochim. Biophys. Acta **465**:290-310.
7. **DePamphilis, M. L., and J. Adler.** 1971. Purification of intact flagella from *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. **105**:376-383.
8. **DePamphilis, M. L., and J. Adler.** 1971. Fine structure and isolation of the hook-basal body complex of flagella from *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. **105**:384-395.
9. **DePamphilis, M. L., and J. Adler.** 1971. Attachment of flagellar bodies to the cell envelope: specific attachment to the outer, lipopolysaccharide membrane and the cytoplasmic membrane. J. Bacteriol. **105**:396-407.
10. **dePetris, S.** 1965. Ultrastructure of the cell wall of *Escherichia coli*. J. Ultrastruct. Res. **12**:247-262.
11. **dePetris, S.** 1967. Ultrastructure of the cell wall of *Escherichia coli* and chemical nature of its constituent layers. J. Ultrastruct. Res. **19**:45-83.
12. **Iino, T.** 1969. Polarity of flagellar growth in Salmonella. J. Gen. Microbiol. **56**:227-238.
13. **Inouye, M.** 1975. Biosynthesis and assembly of the outer membrane proteins of *Escherichia coli*, p. 351-391. *In* A. Tzagoloff (ed.), Membrane biogenesis. Plenum Press, New York.
14. **Martinez, R. J., and E. Z. Gordee.** 1966. Formation of bacterial flagella. I. Demonstration of a functional flagellin pool in *Spirillum serpens* and *Bacillus subtilis*. J. Bacteriol. **91**:870-875.
15. **Moore, H.** 1966. Use of freeze etching in the study of biological ultrastructure. Int. Rev. Exp. Pathol. **5**:179-216.
16. **Murray, R. G. E., and A. Birch-Andersen.** 1963. Specialized structure in the region of the flagella tuft in *Spirillum serpens*. Can. J. Microbiol. **9**:394-401.
17. **Murray, R. G. E., P. Steed, and H. E. Elson.** 1965. The location of the mucopeptide in sections of the cell wall of *Escherichia coli* and other Gram-negative bacteria. Can. J. Microbiol. **11**:547-560.
18. **Remsen, C. C., S. W. Watson, J. B. Waterbury, and H. G. Trüper.** 1968. Fine structure of *Ectothiorhodospira mobilis* Pelsh. J. Bacteriol. **95**:2374-2392.
19. **Snellen, J. E., and M. P. Starr.** 1976. Alterations in the cell wall of *Spirillum serpens* VHL early in its association with *Bdellovibrio bacteriovorus* 109D. Arch. Microbiol. **108**:55-64.
20. **Vaituzis, Z., and R. N. Doetsch.** 1965. Relationship between cell wall, cytoplasmic membrane, and bacterial motility. J. Bacteriol. **100**:512-521.
21. **Weidel, W., H. Frank, and H. H. Martin.** 1960. The rigid layer of the cell wall of *Escherichia coli* strain B. J. Gen. Microbiol. **22**:158-166.
22. **Wells, J. S., and N. R. Kreig.** 1965. Cultivation of *Spirillum volutans* in a bacteria-free environment. J. Bacteriol. **90**:817-818.